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Demonstration of anthrax bacilli after immunization with Carbozoo.

by Christian Troger.

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Translated from: Zeitschrift fuer Immunitätsforschung,  
76: 187-201 (1932).

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Studies of the persistence of virulent pathogens instilled in animals for the purpose of active immunization not only incite scientific curiosity, but are also highly significant from the viewpoint of veterinary and human hygiene, especially in such cases where a transmission of infection from animal to man by consumption of infected meat is within the realm of possibility.

The demonstration of anthrax bacilli in rabbits immunized with Sobernheim culture is treated by Nennings, whose results I should like to recount briefly for purposes of comparison.

Nennings found anthracoid colonies on agar streak cultures or enrichment broth seeded with material from the proximity of the site of injection 8, 15, 20 and 30 hours after instillation of 0.5 cc Sobernheim culture in one rabbit each. Upon simultaneous inoculation of 0.5 cc culture and 5 cc serum, 1 rabbit produced these colonies after 40 hours, 2 rabbits after 3 days, and 1 rabbit 6 days p.i. The diagnosis of anthrax was confirmed by animal test.

The demonstration of precipitinogen in the skin of immunized cattle is discussed in a paper by Kurt Schern of Montevideo, according to which moist dried skins of cattle inoculated against anthrax induce precipitation.

K. Bierbaum, W. Krause and A. Reinstorf were unable to show precipitinogenic substances in cattle skins by means of Ascoli's test. The first two investigators examined aqueous and chloroformic extracts of skin particles from different sites following immunization according to Pasteur; the latter tested chloroformic and cold extracts after Koch's simultaneous inoculation. Schern's positive findings therefore were not confirmed by thorough reexaminations on the part of the authors mentioned above. It is true that only rabbits were used in my tests, resulting in 4 positive cases, as shall be discussed later in more detail.

Nennings' paper contains the following important conclusion:  
"The results obtained in tests with rabbits naturally cannot be applied without reservation to large animals (horses and cattle), since only tests with the latter can answer questions about the serviceability of the meat and hide within a certain time after sero-vaccination against anthrax."

In Austria, the following ordinance of the Federal Ministry for Agriculture and Forestry No. 36.959 - Vt. V. dated 16 November 1928, subject: "Utilization of the meat of sacrificed animals immunized against anthrax," is applicable in this respect:

Animals vaccinated with infectious pathogens of anthrax may be put out to graze or be killed in the first week after inoculation only with the approval of the District police, unless sacrifices or accidents are involved. The following specimens must be sent to testing centers, if the animal has been sacrificed during this period and its meat is intended for human consumption:

- 1) The site of inoculation (a circular piece of skin about 10 cm in diameter and the underlying muscle in natural attachment); the site is recognized by the point of injection which should have been depilated and shaved prior to each vaccination;

- 2) the regional lymph node;

- 3) the spleen and

- 4) one tubular bone.

If no anthrax bacilli are demonstrable in any of these samples, the meat is to be evaluated after a repeated inspection.

In evaluations of the meat of animals butchered after the period indicated above, no consideration of past immunization is necessary, provided the animal does not reveal either a swelling at the site of inoculation, or a swelling of the regional lymph node; if such is the case, however, the release of the meat also depends on the result of a bacteriological examination of specimens sent in according to the aforementioned rules.

The impetus for the present paper came from immunizations carried out in Upper Austria in the summer of 1931 on the occasion of an extensive anthrax epizootic on the mountain meadows of the Alpine foothills. The vaccine utilized was Carbozoo (method Mazzucchi-Milan), an immunogen previously used in the year 1930 by the Federal Institute for the Control of Epizootics at Moedling in several anthracic regions, which had proved to be harmless and very effective in inducing a high degree of immunity, as evident also from previously conducted laboratory experiments. The question was raised on that occasion, how long the

instilled anthrax bacilli survive in the animal organism and whether the inoculated animals expulse the pathogen in their excretions or secretions, and if so, how long. The systematic treatment of these questions is interesting also from the viewpoint of meat inspection, if an animal is involved that must be sacrificed a short time after Carbozoo immunization for accidental reasons, e.g. a bone fracture, as indeed happened in one case.

My tests utilized the original vaccine "Carbozoo" of the Milanese Serotherapeutic Institute, based on Mazzuccini's method.

According to Prof. Pelfanti, this anthrax vaccine contains fully virulent anthrax bacilli subjected to the effects of saponium purissimum album.

#### Personal tests.

The initial task I faced was the systematic examination of rabbits, pre-treated with bovine or ovine doses of Carbozoo vaccine, for presence of anthrax bacilli. In addition, attempts were made to determine the existence of precipitinogen in the test animals, and this in dried particles of skin from the site of inoculation and the nape.

The technique utilized by me in this connection was the following with respect to all animals:

Injection of vaccine (alternately the bovine dosis of 0.25 cc and the ovine dosis of 0.125 cc) was made subcutaneously at the hypogastrium in the region of the right knee fold, after shearing and disinfection of the site. The rabbits were killed by a blow to the neck after measured intervals and prepared in the following manner:

After thorough saturation of the fur to prevent dust formation, a circular cut was made with scissors around the thorax behind the shoulder zone, the skin was then drawn backwards over the trunk up to the metatarsi, exposing the site of injection and the regional lymph gland of the knee fold. Subsequent work was carried out with sterile instruments. One agar plate each was seeded from the site of inoculation, from freshly cut surfaces of the lymph gland, the spleen, liver, kidneys, and from the heart blood. In this operation I grasped the organs with tweezers, removed a particle with the aid of scissors and inoculated the agar surfaces with the organs' cut surface as with a spatula. I shredded the remaining organ with two hooked tweezers and dropped it into a broth tube for enrichment. Similarly, I prepared an agar streak culture and enrichment of the edematous, infiltrated tissue particle from the site of injection that adhered either to the skin or the trunk of the dead rabbit after skinning. The heart blood was removed with a platinum spatula after opening the right ventricle and cultivated on agar-agar and a tube of enrichment broth. A slide smear from the edema of the injection site, the spleen and heart blood was

stained according to Gram. The evaluation of agar plates took place after incubation for up to 48 hours at 37°C; in addition, other agar plates were inoculated with the enrichment broth incubated for 48 hours with the aforementioned material, and inspected following the required incubation.

The precipitation of skin fragments was carried out by the cold extract process according to G. Francke, R. Standfuss, F. Schnauder and F. Muessemeler.

Strongly reduced skin particles were placed in small test tubes, where they were subjected to the effect of a 1% carbol-sodium chloride solution for 24 hours. Precipitation tests were undertaken after agitation and clearing of the extract. A number of turbid specimens had to be centrifuged; the tests were made in Ascoli tubes.

The following methods were resorted to for the differentiation of suspected colonies: Their macroscopic and microscopic appearance, slide smears, transplantation to slant agar and subsequent examination of the condensed water in the hanging drop for bacterial motility, transplantation to blood agar plates for the determination of possible hemolysis and, finally, animal tests. Animal tests utilized white mice that were inoculated subcutaneously with the broth suspension of the questionable culture.

After dissection of the dead mouse, its splenic pulp, heart blood and renal tissue were planted on agar plates, and preparations from the spleen, kidney and heart blood were stained according to Olt.

#### Rabbit tests.

##### 1. Preliminary tests.

Three rabbits pre-treated with 0.25 cc original Carbozoo vaccine (series 1 - prepared January 1931 to January 1932) and killed after 20, 48 and 72 hours, failed to reveal anthrax bacilli in tests carried out as indicated.

##### 2. Main tests.

The main tests utilized 36 rabbits weighing 610 to 2,060 g, immunized alternately with 0.125 and 0.25 cc of original Carbozoo. The table reproduced below gives the dosages of vaccine used in the pre-treatment of this series of animals, as well as the time of their sacrifice, followed immediately by the tests; in addition, positive findings of anthrax bacilli and positive results of precipitation are listed, and the following abbreviations are used: Oe. = edema, Ldr. = regional lymph gland, J. = skin fragment from the injection site, N. = skin fragment from the nape, and S. = death due to coccidiosis and anthracic septicemia. Where no results are indicated, the tests were negative.

The immunized animals revealed the following reactions:

Rabbits inoculated with Carbozoo showed a local reaction at the site of injection in the form of a slight, doughy swelling in the first few hours, becoming more pronounced after about 16 hours. This swelling was found to be a serous or serous-hemorrhagic edema of the subcutis upon section. The serous infiltration was strongly defined up to the 11th day p.i., with preponderance of the hemorrhagic quality up to the 8th day.

No further signs of a reaction were seen in the live animal.

Thirty-four of the 36 immunized animals appeared healthy at the time of sacrifice, animals #21 and 30 died before the scheduled kill due to an intercurrent disease, namely high-grade intestinal coccidiosis. In these 2 cases anthrax bacilli were demonstrated in all organs both in stained smears and culturally, and mice inoculated with organ material of these two rabbits died of anthrax within 3 days.

Hereafter, only the 8 cases with positive findings of anthrax shall be discussed. All remaining animals failed to yield anthrax bacilli by means of the test methods outlined above.

Rabbit 1: The rabbit, inoculated with 0.75 cc orig. Carbozoo series 1 and sacrificed after 4 hours, produced anthracoid colonies on an agar plate seeded with edema. Agar plates inoculated from the enrichment broth yielded anthrax colonies in pure culture. A mouse injected with questionable primary colonies on agar died of typical anthrax 3 days p.i. Agar plates seeded with organ material from other organs of rabbit #1 remained sterile.

Rabbit 2: Immunized with 0.25 cc Carbozoo and killed 4 hours later. Both agar plates of edema and agar plates of the enrichment broth produced pure anthracoid cultures. A mouse died of anthrax 3 days after inoculation of agar culture. The remaining organs proved sterile.

Rabbit 4: This rabbit, injected with 0.125 cc Carbozoo 1 and sacrificed 8 hours later, yielded anthracoid colonies on agar plates seeded with edematous fluid from the site of inoculation. Similarly, agar plates of enrichment broth grew out numerous anthracoid colonies in pure culture. The resultant cultures, instilled in mice, killed these animals within 2 days. Positive anthrax.

Rabbit 14: The regional lymph gland of this rabbit, inoculated with 0.125 cc orig. Carbozoo 1 and killed after 36 hours, produced numerous anthracoid colonies in pure culture, but only on plates seeded with enrichment broth. The other agar plates remained sterile. A mouse injected with this culture died of anthrax after 2 days.



Rabbit 23: The edema taken from the injection site of this animal, immunized with 0.125 cc orig. Carbozoo 1 and killed after 4 days, led to the germination of one anthracic colony on agar plates. A mouse inoculated with a broth suspension of this colony died of anthrax 4 days p.i.

Rabbit 25: This animal, immunized with 0.25 cc orig. Carbozoo 1 and sacrificed 5 days later, produced isolated anthracic colonies on agar plates of the regional lymph gland, and numerous colonies on plates seeded with enriched broth (enrichment consisted of edematous fluid and material from the regional lymph gland). One mouse injected with a suspension of colonies produced by the edema died of anthrax on the 4th day; another, inoculated with suspended colonies from the regional lymph gland, died of anthrax 3 days p.i.

Rabbits 21 and 30 have already been mentioned; they died prematurely of advanced occidiosis with a demonstration of anthrax in septicemic dissemination.

Precipitation tests with extracts from skin fragments of the nape and the site of injection led to following results:

Skin particles from the injection site of the following animals reacted positively: #2, 13, 15, 21, 22 and 30; a piece of skin from the nape of animals 21 and 30 also registered positively. Precipitation of skin fragments was negative in the case of all other animals.

It may be concluded in summary that anthrax bacilli survived at the most until the 5th day in 36 rabbits alternately immunized with 0.125 cc and 0.25 cc original Carbozoo series 1 (prepared January 1931 to January 1932), i.e. in the edematous fluid in 4 cases, in the regional lymph gland in 1 case, simultaneously in the edematous fluid and the lymph gland in 1 case, and in all organs (massive occidiosis) in 2 cases.

#### Tests with cattle and horses.

The experiments with rabbits were followed by attempts to demonstrate anthrax bacilli in edema from the injection sites of horses and cattle immunized with Carbozoo. Five oxen and 2 horses were available for these experiments.

All animals were first inoculated with 0.25 cc orig. Carbozoo series 2 (prepared April 1931 to April 1932). Subsequently the edematous fluid from the injection site was examined 1, 2, 3, 4, 5, 7, 12 and 14 days after inoculation.

The procurement of edematous fluid proceeded as follows:

After disinfection of the injection site with 50% alcohol, a sterilized hypodermic needle was introduced into the edematous swelling. An assistant forced the edematous fluid into the sterile syringe by pressing and pinching, aided by the suction exerted by the drawn piston. Immediately after withdrawal of the needle I allowed the procured fluid to drip onto 2 agar plates and one peptone broth tube. The edematous fluid on the agar plates was then distributed on the medium with a flamed platinum spatula. Two additional agar plates were seeded from the enrichment broth after incubation for 24 hours.

Here, again, the differentiation of anthrax and anthracoid bacteria was accomplished by examination of motility, hemolysis and animal tests. It is noteworthy that Carbozoo cultures on blood agar plates failed to effect hemolysis for 4 days. In these tests I observed 2 microscopically different forms of anthrax colonies on the agar plates. One of them, being by far the most prevalent and called the Carbozoo form by me, had a dry, granular, grayish-brown appearance with a poor tendency to dissemination. The edge of these colonies seems wavy, but almost completely closed.

The second form of colony, growing with more slime, appeared greenish and gelatinous with a yellowish-white, cloudy turbidness in the center. The edge of these colonies seemed to be smooth.

In the case of animal V (ox), I found only the slimy form of colony and only once the Carbozoo form simultaneously next to the slimy colonies on the same plate; I further observed that bacilli from the slimy colony killed mice more rapidly. After injection of a slimy colony into mice, the organs of the sacrificed animals grew out the Carbozoo form in the incubator, and vice versa. In a few cases the Carbozoo form changed to the slimy form of colony after 3 to 4 days in the incubator, but the process was never observed directly. The Carbozoo form had a very slow relative rate of growth.

The differentiation of colonies frequently proved extremely difficult due to the occurrence of these two colony forms, especially so whenever very small slimy colonies with a definitely non-anthrax growth were present.

Virulence tests to which the original Carbozoo vaccine used with cattle and horses was subjected, yielded the following results:

Two rabbits weighing 318 and 350 dkg, immunized with 0.25 cc orig. Carbozoo series 2 (prepared April 1931- April 1932), survived. Two guinea pigs weighing 208 and 260 dkg, inoculated with 0.25 cc from the same vial, died of anthrax after 8 days. Two agar plates were seeded with the original vaccine. A mouse was inoculated with a suspension from the resultant colonies and died of anthrax 5 days later.

The tests with 5 head of cattle and 2 horses proceeded as follows:

The reaction to the inoculum was expressed on the first day after injection by edema measuring about 15 X 15 X 3 cm in all animals, maintaining nearly identical dimensions until the 3rd day. From that day onward a gradual reduction set in, initially to about 10 X 10 X 3 cm. From the 6th to the 10th day the edematous swelling diminished to about 5 X 5 X 2 cm. Four days later only a small peak was visible at the site of injection. Positively reacting test animals gave evidence of low-grade temperature fluctuations.

Test animals I to V are oxen, VI to VII are horses.

#### First day after Carbozoo vaccination.

Ox I: In this case, both the edema from the injection site and the enrichment broth on agar-agar produced anthracic colonies. A mouse injected with the latter died of anthrax 2 days p.i.

Ox II: Here only one agar plate seeded with enrichment broth yielded isolated Carbozoo colonies. A mouse subjected to these died 6 days p.i.

Oxen III and IV as well as horse VI were free of anthrax bacteria on the first day of Carbozoo vaccination.

Ox V: Agar plates seeded with edematous fluid as well as those streaked with edema-enriched broth showed numerous anthracic colonies. Three white mice injected with such colonies died of anthrax: One after 1 day, one after 2 days, the third after 6 days.

Test animal VII (horse): In this case, too, numerous anthracic colonies grew out on agar plates streaked with edematous fluid and on plates seeded with enrichment broth. These cultures, instilled in a mouse, killed the animal 7 days later.

Examinations of the edematous fluid at the point of injection for anthrax bacilli 2 days after inoculation were negative in all 7 animals.

Similarly, anthrax bacilli were demonstrated in the edematous fluid of oxen I, II, IV, V and horse VI 3 days after immunization. Animal VII (horse) failed to yield any edematous fluid on this day. The edema of animal III produced isolated anthracic colonies on agar. A mouse injected with a broth suspension of this culture died of anthrax after 2 days.

The examination of edematous fluid from animals I, II, III and IV was completely negative four days after immunization with Carbozoo. Animal V produced pure cultures of the slimy type and the typical Carbozoo form out of edematous fluid on agar-agar and enrichment broth.

A mouse inoculated with this material died of anthrax 1 day later (slimy colony). Two other mice succumbed to anthrax within 2 days (both showed the Carbozoo form in cultures).

Test animal VI (horse): The edema produced a single anthracoid colony on agar which, in the form of a broth suspension, killed a subcutaneously inoculated mouse after 2 days.

Test animal VII also produced only a single colony of anthrax bacilli on an agar plate seeded with edematous fluid, causing typical anthrax in a subcutaneously injected mouse after 1 day.

In examinations of edematous fluid from the injection site of the animals 5 days after immunization with Carbozoo anthrax bacilli could not be demonstrated in test animals I, II, III, IV, VI and VII. Only animal V yielded slimy, smooth-edged colonies on agar plates seeded with edematous fluid which, when subcutaneously instilled in a mouse, caused the animal's death after 1 day. Organ material of this mouse grew out slimy colonies of Carbozoo on agar plates.

Examinations of edematous fluid could not be made on the 6th day after immunization.

Tests of edematous fluid of animals I, II, III, IV and V were completely negative 7 days p.i.

Test animal VI (horse) produced colonies both of slimy and Carbozoo type on agar plates seeded with edema from the site of injection; plate cultures of enriched broth yielded only those of the slimy form. Two white mice inoculated with such cultures died of anthrax 1 day later.

Test animal VII (horse) gave positive results in the form of colonies growing on agar plates seeded with enriched broth; one plate grew out slimy colonies, the other Carbozoo forms. A mouse injected with the culture suspension died of anthrax 4 days p.i.

Examination of the edematous fluid at the injection site of both horses (VI and VII) 12 days after immunization with Carbozoo led to a negative result in the case of animal VI, while agar plates of animal VII showed 1 anthracoid colony on one plate and 3 colonies on the other. The infected mouse succumbed to anthrax one day later. The oxen were not tested on this day.

On the 14th day after vaccination with Carbozoo I examined the edematous fluid from the injection site of all 7 animals for the last time. By this time it proved extremely difficult to obtain edematous fluid, especially in the case of oxen, since the reaction had almost entirely disappeared. The tests were negative without a single exception.

The following table consolidates findings of anthrax bacilli in the injection site of cattle and horses inoculated subcutaneously with Carbozoo.

The table reveals that anthrax bacilli were found in the edematous fluid of oxen 5 days after vaccination, whereas the two horses yielded the pathogen for up to 12 days.

Examinations of milk and urine for the presence of anthrax bacilli in cows immunized with Carbozoo.....

Tests aimed at demonstration of anthrax bacilli in edematous fluid from the injection site of Carbozoo-vaccinated cattle and horses were followed by examinations of the milk of 3 cows and the urine of 2 cows immunized with Carbozoo. The tests covered the 1st, 2d, 3rd, 4th, 6th, 7th, 8th, 9th and 10th day after inoculation with Carbozoo, carried out in dosages of 0.25 cc original Carbozoo series 1 (prepared January 1931 - January 1932).

In addition, these 3 cows were subjected to tests of edematous fluid from the injection site on the 6th, 8th, 9th, 10th and 11th day after inoculation, days on which previous examinations of edema among the cattle and horses had not been accomplished.

The test procedure was the same for all animals:

After thorough disinfection of the teats with 50% alcohol, the required quantity of milk was drawn off from all quarters under conditions of greatest cleanliness. After 15 minutes of vigorous centrifugation a sample of sediment was removed with a flared platinum loop, a slide smear was prepared and stained according to Gram. One agar plate and a broth suspension were prepared, the latter for purposes of enrichment. The enrichment broth, incubated for 24 hours, was subsequently transferred to agar plates. The remaining sediment was used for broth suspensions and inoculation of mice.

I obtained the urine by catheterization under maximal conditions of cleanliness, after cleaning the vaginal vestibule with a weak potassium permanganate solution. A major portion of the urine was first allowed to flow off. The urine was then examined in the manner described for milk.

All test aimed at the demonstration of anthrax bacilli in the milk, urine and edema at the injection site of these 3 cows were negative.

Dolezel had arrived at the same result in 1914 during systematic daily examinations of the milk of 3 cows and 2 goats following Pasteur-immunization, extending over 12-23 days.

#### Summary.

Eight out of 39 rabbits (20.51%) sacrificed after pre-treatment with bovine or ovine doses of Carbozoo vaccine yielded anthrax bacilli in the edema at the injection site in 4 cases (in 2 cases after 4 hours, in one case after 8 hours, in another after 4 days), in the regional lymph gland in one case (after 5 days) and in all organs in 2 cases (after 4 and 8 days, respectively). In the last 2 cases the rabbits had succumbed to advanced coccidiosis prior to the planned sacrifice and revealed anthrax bacilli in septicemic dissemination.

The remaining animals (79.49%), tested a few hours to 14 days after inoculation with Carbozoo, yielded no anthrax bacilli either at the injection site, in the regional lymph gland, or in the spleen, liver, kidneys and the heart blood.

The demonstration of precipitinogen in the dried skin of rabbits apparently healthy at the time of sacrifice succeeded in 4 cases, 4 and 36 hours and 2 and 4 days after inoculation, respectively, but only in extracts prepared from skin particles from the site of injection. (Cold extraction method according to Francke, Standfuss, Schnauder and Muessemeier).

The two rabbits (21 and 30) succumbing to coccidiosis and anthracic septicemia 4 and 8 days after vaccination gave a positive precipitin reaction with skin fragments from the injection site and the nape.

Of 5 oxen and 2 horses immunized with original Carbozoo, 3 oxen and 1 horse yielded anthrax bacilli in the edema 1 day after inoculation, 1 ox 3 days p.i., 1 ox and both horses 4 days p.i., 1 ox on the 5th day, both horses on the 7th day and only 1 horse on the 12th day following injection, whereas no anthrax bacilli could be demonstrated in the other animals on these days, nor could they be found in any of the animals on the 2d and 14th day after immunization.

The occurrence of anthrax bacilli in the milk and urine of 3 cows immunized with Carbozoo could not be observed on the 1st, 2d, 3rd, 4th, 6th, 7th, 8th, 9th and 10th day p.i. Nor did supplementary tests produce the pathogen from the injection sites of these 3 cows on the 6th, 8th, 9th, 10th and 11th day.

Based on these results, gained from experiments with large animals, the ordinance of the Federal Ministry for Agriculture and Forestry Z. 36.959 - Vt. V dated 16 Nov. 1928, subject: "Utilization of the meat of sacrificed animals immunized against anthrax," the period of one week following vaccination specified for the submission of specific organs and organs parts for bacteriological examination, should be extended to 2 weeks, preferably to 3 weeks.